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(54) Production of polymetric beads having alginate shells

(57) Beads comprising an outer alginate shell of millimeter-size range and a core of a biologically active material in a liquid or in a gel, or of an oily substance are obtained by supplying to a common coaxial outlet port an inner stream of the core material, a median stream of an alginate solution and an outer stream of a gas to form drops. The drops fall into a solution adapted to coagulate the alginate and are left in the solution until the shell hardens. In a preferred embodiment, the core material comprises a prepolymer, especially polyacrylamide-hydrazine which is polymerized after hardening of the shell, which shell may subsequently be removed.

The invention also provides a device for forming the beads. This device comprises three fluid conduits adjustable for individual flow rates and respectively supplying the alginate solution, the core material and the gas stream to the common coaxial outlet port. Preferably, said conduits are coaxial needles.

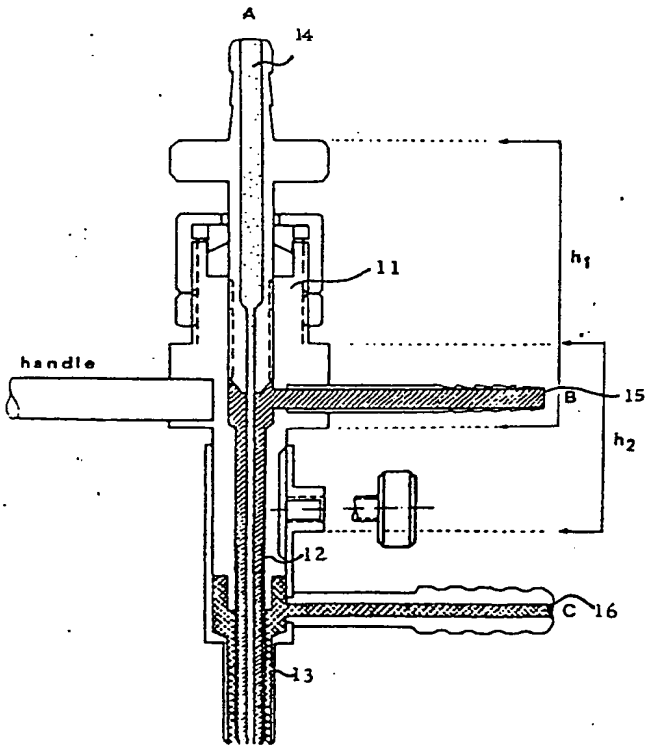

Fig. 1

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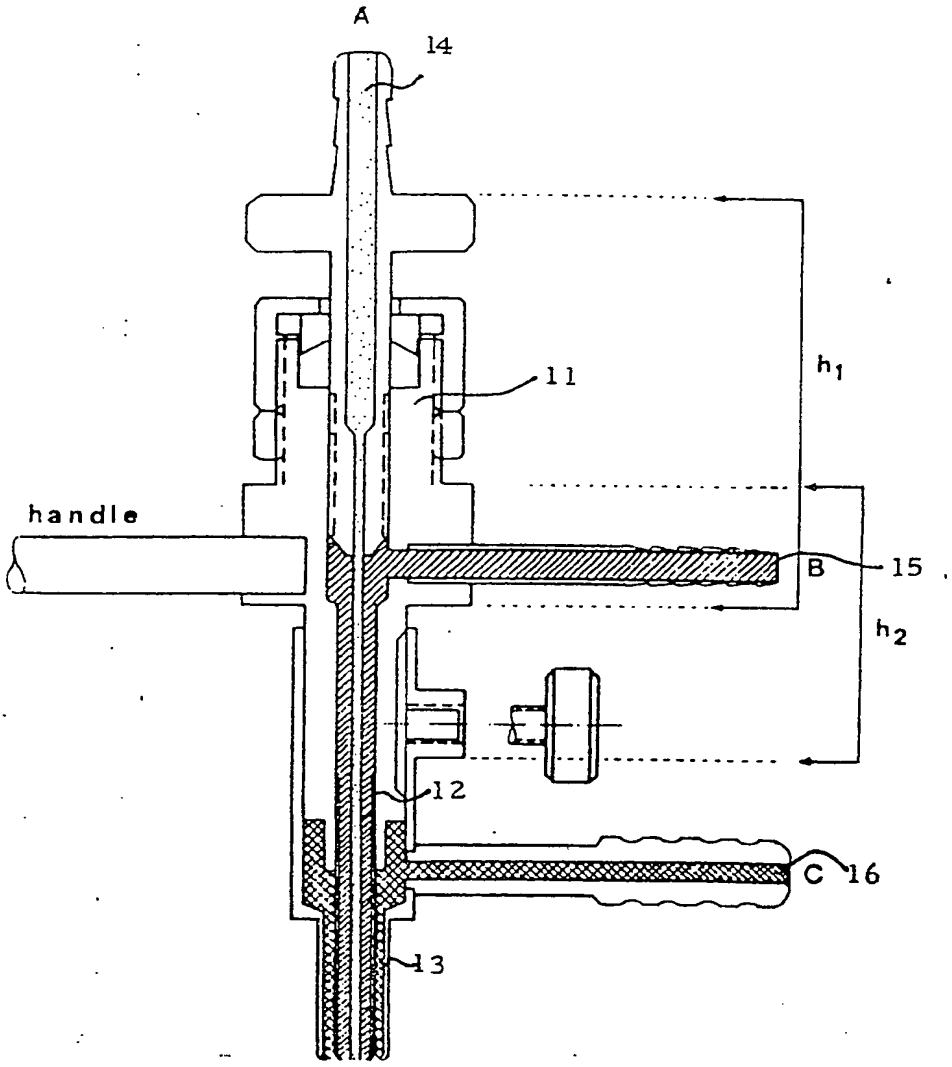


FIG. 1

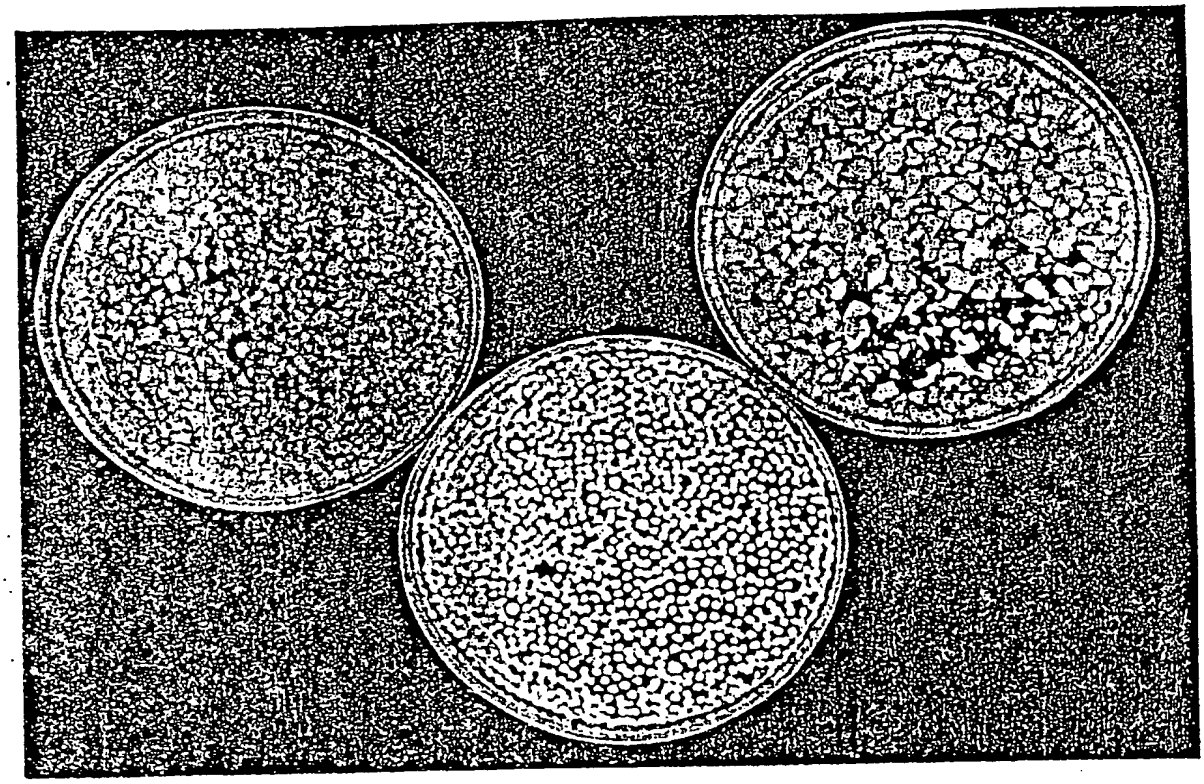


FIG. 2

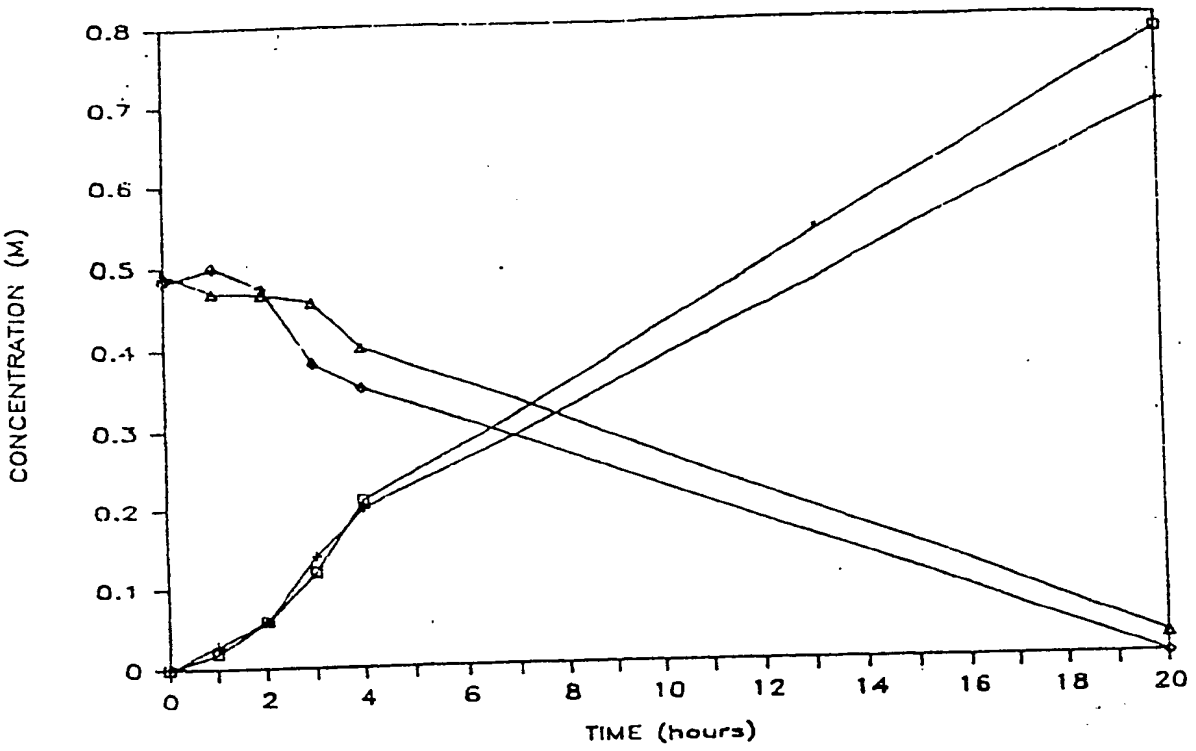
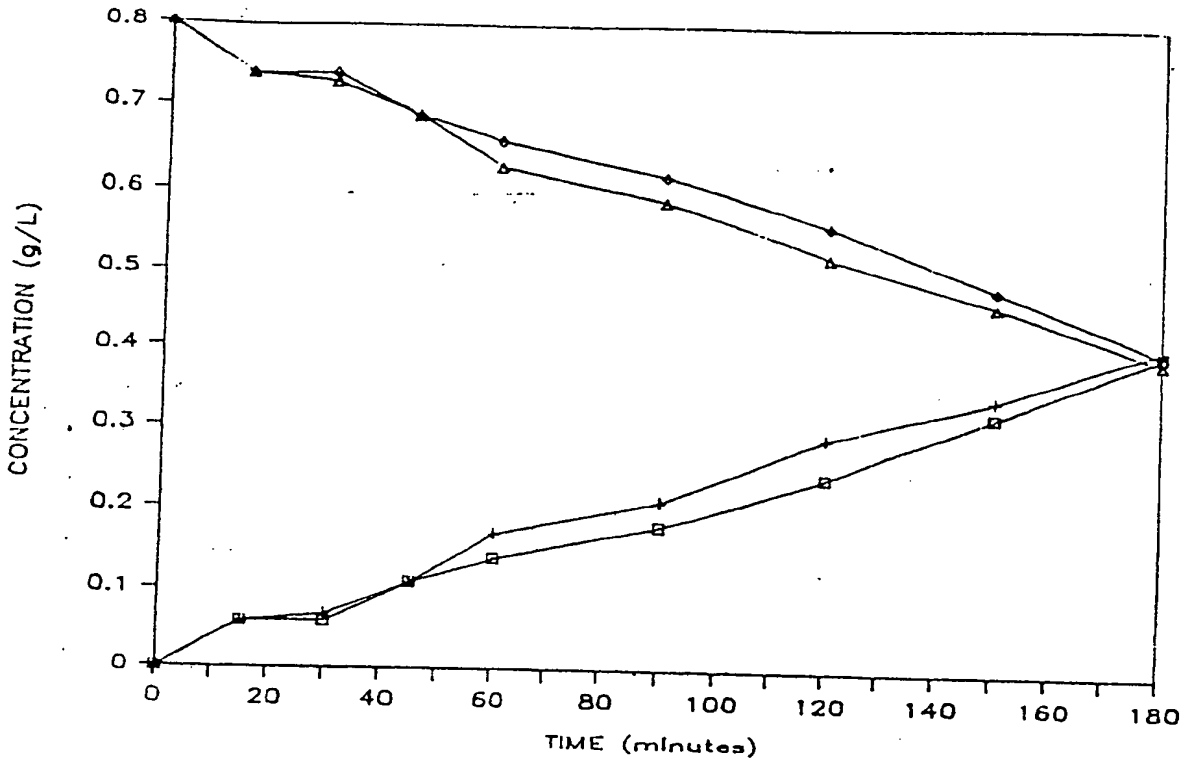


FIG. 3

500mg of cells were immobilized and assayed in parallel with freely suspended cell control at 30°, 150 rpm, employing 10% (w/v) glucose in 0.3% citrate buffer, pH 5, as substrate (50 ml)

(Δ) glucose consumption by freely suspended yeasts;
(◊) by immobilized cells;
(+) ethanol production by freely suspended yeasts;
(□) by immobilized cells.

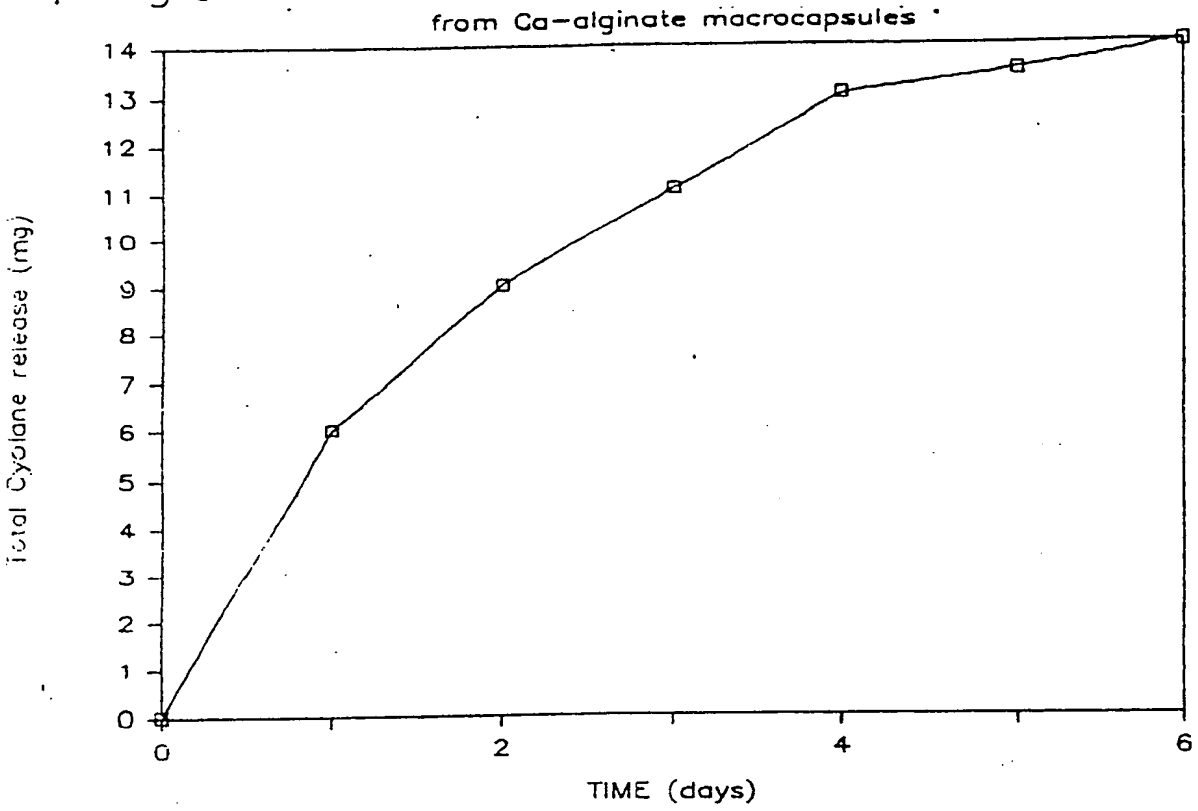
FIG. 4



Forty mg (ww) of cells were immobilized and assayed, in parallel with freely suspended cells control, at 30°, 150 rpm, employing 0.8 g/l hydrocortisone solution in 8% (v/v) ethylene glycol in 0.05 M tris buffer, pH=7.8 (50 ml).

(Δ) Hydrocortisone consumption by freely suspended cells;
(◊) by immobilized cells;
(+) Prednisolone production by freely suspended cells;
(□) by immobilized cells.

Fig.5: Total amount of Cyolane release



SPECIFICATION

Production of polymeric beads

The invention relates to a device for the production of alginate beads which can be produced in an essentially uniform size. The beads comprise an outer alginate shell and a core which can consist of a liquid or gel or water-in-oil emulsion, said gel or liquid containing entrapped cells in suspension, biologically active particles in suspension. The core can also consist of an oil or oily substance which may also contain a biologically active material.

If desired the alginate shell can be subsequently removed. The beads can be produced in the millimeter-size range, and there can be produced beads within a desired predetermined essentially uniform size range.

The device of the invention comprises 3 conduits through which there flow an alginate solution, a solution or suspension subsequently forming the core of the bead and a gaseous stream. The resulting beads are dropped into a solution adapted to solidify the alginate shell of the beads, which are subsequently washed. The active material can be present in a prepolymer, and in such case the bead can be contacted after hardening of the alginate shell with a medium adapted to polymerize the prepolymer. Although mainly intended for the entrapment of cells and other biologically active material in millimeter-size beads, the same alginate shell can also be produced to contain an oil, such as vegetable oil, paraffin oil, or suspensions or emulsions based on a hydrophobic water-insoluble medium.

The immobilization of whole cells has gained much attention in recent years. Several methods for whole cell immobilization were developed (for review see J. Klein and F. Wagner, App. Biochem. Bioeng. 4, 12-51, 1983). The advantages inherent in immobilized cell systems, such as reuse of biocatalyst, flexibility in reactor design and significantly improved operational stability, led to the development and use of immobilized cell systems on bench, pilot and industrial scale (for review see P. Linko & Y. Linko, CRC Critical Reviews in Biotechnology, 1, 289-338, 1984).

The most popular approach to cell immobilization is by gel entrapment. Cells are suspended in an aqueous solution of a monomer or prepolymer; by physical or chemical change (e.g. cooling, pH change, introduction of polymerization initiator) the solution is gelified, physically entrapping the cells.

In recent years, gel entrapment of cells by crosslinking of synthetic prepolymers gained much attention (S. Fukui & A. Tanaka, Ad. Biochem. Eng. 29, 2-33, 1984). A novel gel entrapment technique, based on chemical crosslinking of linear, water soluble polyacrylamide, partially substituted with acylhydrazide groups, was developed in our laboratory.

The method is based on suspending cells, organelles or enzymes in a solution of polyacrylamide-hydrazide (PAAH) and crosslinking by the addition of controlled amounts of dialdehyde (e.g. glyoxal).

Following hardening, the resulting gel block is mechanically fragmented. This technique was found useful for the immobilization of bacteria, yeast, plant cells, liver microsomes and enzymes (for review see A. Freeman, Annal. N.Y. Acad: Sci. 434, 418-426 1984). The advantages of this technique include retention of activity (90% and more of input activity), high porosity, good chemical, biological and mechanical stability and stabilization of entrapped cells towards organic solvents.

There is provided a device for the production of beads of essentially uniform size, in the millimeter-diameter range, comprising an outer alginate shell and containing a cell suspension, or cells entrapped in a gel, or a biologically active material, such as enzyme, antibodies, immobilized on a suitable carrier, within such shell.

The device provided also enables the preparation of macrocapsules comprising an alginate outer shell retaining droplets of hydrophobic moiety such as vegetable oil, paraffin oil, or solutions, emulsions or suspensions based on hydrophobic, water immiscible medium.

There is further provided a process for the production of such alginate beads. Other and further objects of invention will become apparent hereinafter.

The invention is described by way of illustration with reference to the Drawings, in which:

Figure 1 is a longitudinal sectional schematical view, not according to size, of a device according to the invention;

Figure 2 is a comparison of size distribution of PAAH-entrapped yeast preparations: top right: previous procedure, one fragmentation; top left: previous procedure, two fragmentations; bottom: bead preparate;

Figure 3 is a graph illustrating the conversion of glucose to ethanol by freely suspended, and by PAAH bead-entrapped Saccharomyces cerevisiae;

Figure 4 is a graph illustrating the Δ^1-dehydrogenation of hydrocortisones by free and PAAH bead-entrapped Arthobacter simplex; and

Figure 5 illustrates the amount of Cyolane release from Ca-alginate beads of the invention over a period of a number of days and the rate of release.

Essentially the device according to the invention comprises 3 conduits terminating at a common exit port, one of these supplying a cell suspension or a suspension of small particles with attached biologically active material, in a liquid medium which can be a polymerizable prepolymer; the second conduit supplying an aqueous alginate solution, and the third a stream of a gaseous medium, the flow of each of these being adjustable at will. Preferably an inner conduit supplies the cell or particle suspension. According to a preferred embodiment of the invention, the three conduits are in the form of three concentric tubular members, the

inner supplying the suspension, the second the alginate, and the third the gaseous medium.

The exact distance of the outlets respective each other can be adjusted, thus permitting, together with the adjustment of the flow of each of the three media, to obtain beads of predetermined size, shell thickness and production rate.

The injector device according to the invention is illustrated by way of example with reference to the enclosed schematical drawing in Figure 1.

As shown in Figure 1, the injector comprises three coaxial tubular members 11, 12 and 13, termed hereinafter as "needles", where 14 designates the inlet of the cell suspension, 15 the inlet of the alginate solution and 16 the inlet of the gas stream. Each of these can be adjusted at will as regards flow velocity and volume. The distance of the needle tips defining the outlets can be adjusted at will by varying the distances h_1 and h_2. The experimental unit illustrated has an overall length of about 5 - 10 cm and the exact dimensions of the components is indicated hereinafter.

It is clear that this is by way of illustration only, and various modifications in the arrangement and size of parts can be resorted to.

The PAAH bead entrapped cells may be readily prepared in large quantity, under sterile and anaerobic conditions, in a continuous process. The head entrapped cells have the advantages inherent in the PAAH technique: retention of high activity, high porosity and good stability. The beads are quite uniform and can be adjusted to be within the range of 1 - 5 mm in diameter; the alginate layer occupies ~ 5 - 10 % of the total volume. The inner needle is supplied with a controlled feed of a cell suspension in PAAH (or other solution) through entry port 14. This needle is positioned in a needle of large diameter, which is supplied with a sodium alginate solution through entry port 15. The two needles are held in the center of a third coaxial needle, which is supplied with a controllable stream of air or other gas via entry port 16. The exact distances between the needles may be adjusted by varying the distanced h_1 and h_2. The unit described is about 5 - 10 cm in length and is held mechanically in an upright position.

By adjusting the flow rate of the cell suspension, alginate solution and gas, droplets comprising an inner core, originating from 14, encapsulated in an outer shell coming from 15, are formed; these are detached and spun down by the gas stream from 16, these are of quite uniform size. When an alginate solution is supplied via 15, the shell is gelified by contact with a $CaCl_2$ solution. The beads drop from the injector into a $CaCl_2$ solution and are left for hardening; and the medium is changed to a glyoxal containing buffer. The glyoxal penetrates the alginate layer and thus the inner PAAH solution is crosslinked. Following washings, the beads are ready for use. The process allows immobilization of a wide variety of cells in uniform size, chemically stable, crosslinked polymeric beads.

In a similar way, by feeding oils, oil-based solutions, emulsion or suspension in oils through 14, macrocapsules comprised of caalginate outer shell and filled with oil-based internal droplet are formed.

Example 1

Glucose conversion into alcohol by immobilized saccaromyses cerevisiae in PAAH - beads

a. *Preparation of solutions:*

1. *Cell suspension:*

Into a 5% (w/v) aqueous solution of polyacrylamide-hydrazide (mean average molecular weight of 100,000 containing 0.8 meq. acrylhydrazide groups), a preweighed amount of yeasts, as obtained from the producer "Paka" Industries Ltd., Bat-Yam, Israel, in a ratio of 0.5 g (w/v) of yeasts to 20 ml of gel forming reaction mixture, was added, and the suspension was homogenized by magnetic stirring for 15 minutes at room temperature.

2. *Sodium alginate solution:*

3% (w/v) solution was prepared by dissolving sodium alginate (BDH) in water by magnetic stirring overnight at room temperature. Prior to use this solution was deaerated under vacuum.

b. *Injection system: Specifications (see Figure 1)*

Dimensions and flow rates :

1. Inner tube:	
inner diameter	-(A) - 0.35 mm - 2.0 mm
flow rate	- 0.7 - 0.9 ml/min
2. Median tube - (B): inner diameter	1.2 mm - 3.0 mm
flow rate	- 0.5 - 0.7 ml/min
3. External tube (C): inner diameter	2.0 mm - 5.0 mm
flow rate	2.5 - 4.0 ml/min

Distances between tube outlets:

h_1	2.0 - 2.2 cm
h_2	2.2 - 2.5 cm

c. *The flowing-through tubes A,B and C:*

1. Via tube A: cells suspended in an aqueous solution of polyacrylamide-hydrazide (3 - 5% (w/v)) or in an appropriate buffer solution.
2. Via tube B: sodium alginate solution (BDH, 2-4% (w/v)).
3. Via tube C: nitrogen or air.

d. *Immobilization of yeasts in PAAH - beads*

The homogenized yeast suspension was passed through the inner tube (A) of the system at the flow rate indicated above. Alginate solution and air were supplied at the rates given. The formed drops were collected in a 1% (w/v) aqueous $CaCl_2$ solution and after 1 h incubation this solution was replaced by 50 ml of phosphate buffer (50 mM, pH = 7) containing 2 ml of 5% glyoxal solution (in 50 mM phosphate, pH = 7). The PAAH core was allowed to undergo gelation by incubation overnight at 4°C. The beads were then washed with cold 0.3% (w/v) citrate buffer, pH=5.

e. *Assay*:

Gel beads (1.5 - 2.0 mm in diameter) containing 0.5 g (w/v) of entrapped yeasts were washed three times with 50 ml of cold medium containing 10% (w/v) glucose; 0.15 (w/v) yeast extract; 0.25% (w/v) NH_4Cl; 0.55% (w/v) KH_2PO_4; 0.025 (w/v) $Mg.SO_4.7H_2O$; 0.01% (w/v) NaCl; 0.001% (w/v) $CaCl_2$ and 0.3% (w/v) citric acid, pH = 5.

Finally, the same medium was added to a final volume of 50 ml. This last reaction mixture was then transferred into a 125 ml baffled Erlenmeyer flask that was shaken in a reciprocal shaker at 100 strokes/min. at 30°C allowing the formed CO_2 to escape through a pinhole outlet.

Samples of 1 ml were withdrawn for ethanol and glucose determinations up to 20 h. Under these conditions full conversion of glucose into ethanol was observed within 20 h of incubation.

Ethanol determination:

The concentration of ethanol in samples was determined by G.C.

Glucose determination:

The concentration of glucose was assayed by the "Glucostat" kit of Sigma.

Under the conditions employed, glucose conversion by the PAAH bead entrapped yeast was the same as that of the freely suspended yeast control (see Figure 2).

Example 2

Δ^1-*Dehydrogenation of hydrocortisone by PAAH - bead entrapped Arthrobacter simplex cells*

a. *Cell growth and harvest*

1. Arthrobacter simplex colony (ATCC 6946) was scraped from slant agar into 30 ml of 8 g/l nutrient broth (Oxoid CM 15) sterilized solution in a 250 ml closed universal bottle and incubated for a period of 48 h at 30°C on a gyratory shaker (150 rpm). Into a 2.5 l baffled Erlenmeyer flast 2.5 g yeast extract, 0.75 g $(NH_4)_2SO_4$, 0.66 g $K_2HPO_4 \cdot 3H_2O$ were added, dissolved in 225 ml water, 0.25 ml of trace element stock solution consisting of:

$ZnSO_4.7H_2O$	1.44 g/l
$MMSO_4.4H_2O$	1.12 g/l
H_3BO_3	0.31 g/l
$CuSO_4.5H_2O$	0.63 g/l
$Na_2Mo)_4.2H_2O$	0.25 g/l
$CoCl_2.6H_2O$	0.24 g/l
KI	0.42 g/l
$FeSO_4.7H_2O$	2.78 g/l
H_2SO_4	1-2 ml

were added and the final solution sterilized (121°C, 15).

In parallel, 3.75 g glucose and 25mg $MgCl_2$ were dissolved in 25 ml H_2O sterilized and added to the above solution.

One ml of the cell suspension was transferred into the flask and the flask was incubated for a period of 24 h at 30°C on a gyratory shaker (210 rpm). Five ml of this was used to innoculate in a 2.5 l baffled Erlenmeyer, in the same medium following incubation for 16.5 h at 30°C on a gyratory shaker. After this incubation, 0.25 g of hydrocortisone, finely suspended (magnetic stirring overnight) in 5 ml of 2.5% (w/v) Tween 80 in water were added. Induction was allowed to proceed for 6.5 h.

Cells were collected by centrifugation, frozen in liquid nitrogen and stored at −20°C.

2. *Cell suspension*

Prior to use, a weighed quantity of frozen cells was tranferred to ice cold 0.05 M tris, pH = 7.8 (0.1 g WW (20 mg dcw) cells per ml) and magnetically stirred over ice for 30 minutes. The suspension was then homogenized by a glass-Teflon homogenizer to ensure the formation of a uniform suspension.

b. *Immobilization of cells in PAAH-beads*

Into 10 ml of 5% prepolymer (100,000 MW) solution, equipped with a magnetic stirrer and cooled over ice, 0.4 ml of cell suspension was added, following homogenization of the cell suspension by magnetic stirring. The fine prepolymer cell suspension was then processed to make PAAH-beads as in Example 1. After overnight gelation in 0.05 M phosphate buffer (pH = 7.8, containing 2 ml 5% glyoxal solution), the PAAH-beads entrapped cells were washed three times with cold 0.05 M tris buffer (pH = 7.8) and brought to a final volume of 40 ml with the same buffer.

c. *Assay*

40 g of hydrocortisone was dissolved in 4 ml ethylene glycol followed by the gradual addition of 4 ml water and 2 ml of 0.25 M tris pH = 7.8. The substrate was then transferred into a 250 ml baffled Erlenmeyer flask containing the abovementioned PAAH-bead entrapped cells. 0.05 M tris buffer, pH = 7.8 was added to bring the final volume to 50 ml. The flask was then incubated in a gyratory shaker (30°C, 150 rpm).

The substrate and product were extracted with CH_2Cl_2 and analyzed by HPLC. Under these conditions, 50% conversion of hydrocortisone to prednisolone was observed within 3 hours; (see Figure 4).

Example 3

Beads containing oil droplets:

(1) By applying liquid paraffin oil (Cat. No. 29437, BDH, Poole, England) at a flow rate of 4.5 ml/min through tube A and 3% (w/v) sodium alginate solution (BDH) at a flow rate of 12 ml/min through tube B, and nitrogen at a flow rate of 6 l/min through tube C, and 2% (w/v) $CaCl_2$ in tap water, paraffin oil droplets encapsulated in a Ca-alginate shell were obtained.

(2) In a similar way, by applying corn oil through A (0.4 ml/min) 3% sodium alginate through B ($^{2.0}$ ml/min) and nitrogen through C (10 l/min), corn oil droplets encapsulated within Ca-alginated shell were obtained. In a similar way, β-carotene solution in corn oil was encapsulated within Ca-alginate shell.

Example 4

Preparation of controlled release formulation for systemic insecticides

By applying "Cyolan" (2-(Diethoxyphosphinylimino)-1,3-dithiolane). (American Cyanamid Co., USA) 10 mg/ml solution in corn oil-l-dodecyl alcohol (Cat. No. 28276, BDH) 40:60 (v/v) solution (at 40°C) at a flow rate of 0.4 ml/min through A, and 3% sodium alginate solution through B (2.0 ml/min) and nitrogen through C (10 l/min), solid Cyolan solution in corn-oil-dodecanol, encapsulated within an alginate shell could be obtained (in 3% $CaCl_2$ solution in tap water).

Following 1 hour incubation for hardening in $CaCl_2$ solution the beads were transferred into methanol for 5 min and then into hexane for 1 min. The beads were separated by filtration and 8 gr of beads (WW) were placed in 50 ml distilled water in a glass beaker. Samples were withdrawn daily, followed by washing of the beads with 20 ml of water and reincubation in fresh 50 ml distilled water. Cyolan content in water was determined by HPLC. Samples (5 μl) were assayed on a 12.5 × 0.4 cm Lichrochart RP-8 (4 μ) column (Merck, Darmstadt, FRG) employing methanol-water (60:40) as mobile phase at a rate of 0.6 ml/min (retention time: 4 min). Standards and samples were detected at 245 nm. Cyolan release profile under these conditions is shown in Figure 5.

CLAIMS

1. A process for the production of millimeter-size alginate beads comprising an alginate shell and a core of a biologically active material or of an oily substance, which comprises simultaneously supplying to a common coaxial outlet port an inner stream of core material, a median stream of an alginate solution and an outer gas stream to form drops, and dropping the said drops into a solution adapted to coagulate the alginate and leaving the beads in said solution to harden the shell.

2. A process according to Claim 1, wherein the core material is a suspension of cells, organelles or biologically active particulate material in a solution of a prepolymer which is subsequently polymerized.

3. A process according to Claim 2, wherein the prepolymer is polyacrylamide-hydrazide (PAAH), which is subsequently cross-linked by means of glyoxal.

4. A process according to any one of the preceding claims wherein the biologically active material is selected from bacteria, fungi, yeast cells, plant cells, enzymes and antibodies.

5. A process according to any one of the preceding claims wherein the active material is entrapped in a polymer matrix and the alginate shell is subsequently removed.

6. A process for the production of beads of millimeter size-range of essentially uniform size, comprising a core of biologically active material in a suspension medium or in a polymer matrix confined in an alginate shell, substantially as hereinbefore described.

7. Beads of essentially uniform size in the millimeter range, containing a core of biologically active material and an outer alginate shell, whenever obtained by a process as claimed in any one of the preceding claims.

8. Millimeter-size alginate shell beads containing a core of a suspension of a biologically active protein or cells or of such material entrapped in a polymer matrix which does not impair the biological activity of such

materials, substantially as hereinbefore described.

9. A device for the production of millimeter-size beads comprising an alginate shell and a liquid or gel-form core of biologically active material or of an oily substance, which device comprises three fluid conduits with individually adjustable flow rates means for adjusting the distance of these respective each other at a common coaxial outlet port, the inner conduit serving as conduit for the material forming the core content, the second conduit being that of the alginate solution and the third being a gas conduit.

10. A device according to Claim 9, wherein the conduits are in the form of three coaxial tubular members having a downwardly pointing common outlet.

11. A device for the production of alginate beads defined in Claim 10, substantially as hereinbefore described with reference to Figure 1.

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